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Identification of *Dll1* (*Delta1*) target genes during mouse embryogenesis using differential expression profiling

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Abstract

The Notch signaling pathway has pleiotropic functions during mammalian embryogenesis. It is required for the patterning and differentiation of the presomitic and somitic paraxial mesoderm and of the neural tube. We used DNA-chip expression profiling and 2D-gel electrophoresis combined with peptide mass fingerprinting to identify genes and proteins differentially regulated in E10.5 *Dll1* (*delta-like 1*, *Delta1*) mutant embryos. The differential expression profiling approach identified 47 regulated transcripts and 40 differentially expressed proteins. The majority of these genes has until now not been associated with Notch signaling. Subsequent whole-mount in situ hybridization confirmed that a subset of the identified transcripts has restricted and distinct patterns of expression in E10.5 mouse embryos. For most genes these expression patterns were affected in the presomitic mesoderm, in differentiating somites of *Dll1* mutant embryos and in the neural tube and cells differentiating from it. Similar effects were observed in embryos homozygous for the *Headturner* (*Htu*) and *pudgy* (*pu*) mutations, which are alleles of the Notch ligands *Jag1* and *Dll3*. The regulated expression of a subset of the proteins was validated by immunoblots. Remarkably six of the proteins down-regulated in *Dll1* mutant embryos are proteasome subunits. The large set of regulated genes identified in this differential expression profiling approach is an important resource for further functional studies.

Keywords: Notch pathway; *Dll1*; *Delta-like 1*; *Delta1*; *Dll3*; *Delta-like 3*; *Delta3*; Pudgy; *Jag1*; Headturner; Embryogenesis; Somitogenesis; Neurogenesis; Mouse; DNA-chip; Microarray; Expression profiling; Transcriptome; Peptide mass fingerprinting; 2D-gel electrophoresis; Proteome; Gene regulation; Regulation of expression

1. Results and discussion

The evolutionary conserved Notch signaling pathway is required for cell differentiation and patterning processes during mammalian embryogenesis including lateral specification during neurogenesis (Beatus and Lendahl, 1998; Morrison et al., 2000; Yun et al., 2002), determination of left and right body halves (Krebs et al., 2003; Przemeck et al., 2003; Raya et al., 2004), patterning of the presomitic mesoderm (Dale et al., 2003; Jouve et al., 2000; Takahashi et al., 2003) and establishment and maintenance of somite boundaries (Barrantes et al., 1999; Hrabe de Angelis et al., 1997). The classical model of Notch signaling cannot explain these developmental processes satisfactorily. Cross-regulation exists, for example, between the Notch and Wnt pathways during patterning of the presomitic mesoderm (Aulehla et al., 2003; Hofmann et al., 2004). The *Dll*1 (*delta-like 1*, *Delta1*) gene encodes one of at least five known ligands of Notch receptors in mice (Bettenhausen and Gossler, 1995; Bettenhausen et al., 1995). To identify novel Notch signaling targets on the RNA and protein levels, we screened the differential transcriptome and proteome of *Dll1*-deficient (*Dll1*^{tm1Gos}) and wild-type (wt) embryos at day 10.5 post coitum (E10.5, Theiler stage 17–18). Expression of candidate genes was validated by in situ hybridization of wt and *Dll1*, *Dll3* and *Jag1* mutant embryos or Western blot analyses, respectively.

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1.1. DNA-chip expression profiling of Dll1 mutant embryos

To identify genes differentially expressed between wt and homozygous *Dll1*^{tm1Gos} embryos at E10.5, DNA-chip expression profiling was performed. We used our custom-made 20 k DNA-chip (mouse arrayTAG) and a commercial 1 k microarray (Atlas Glass Mouse 1.0). The specificity of the fully sequenced probes used on the 20 k DNA-chip was assessed experimentally (Drobyshev et al., 2003) and the chip was used previously for the identification of biologically relevant gene expression (Beckers et al., 2005; Seltmann et al., 2005). For the 20 k microarray 4 and for the 1 k microarray 9 competitive dual-colour hybridizations including dye-swap controls were done. 1815 and 460 probes with significant signals on all 4 and 9 hybridized arrays were analyzed for reproducible expression ratios in all experiments. In total 21 genes were reproducibly up-regulated and 26 genes were down-regulated in homozygous *Dll1*^{tm1Gos} mutant embryos (fully listed in Supplemental Table 1a, b).

In addition, we individually examined expression of 72 genes associated with Notch signaling in general or described as regulated in *Dll1*^{tm1Gos} mutant embryos. Thirty of the seventy-two genes were represented by at least one probe on the mouse arrayTAG DNA-chip and 34 genes were represented by one probe on the Atlas Glass Mouse 1.0 microarray. Forty-nine of the seventy-two genes were represented on at least one of the two DNA-chip platforms. Probes for genes such as *Dll1*, *Tcf15* (*paraxis*), *Scx* (*scleraxis*), *Hes1* and *Jag1* indicated down-regulation and *Neurod4* (*Math3*) was up-regulated (Barrantes et al., 1999; Beckers et al., 2000; Hatakeyama et al., 2004; Jouve et al., 2000). However, hybridization with these probes did either not result in sufficiently strong signals on all chips or were not among the top 47 regulated genes with probability $p < 10\%$ to contain one or more false positive genes. Other *Dll1* target genes, such as *Uncx*4.1, *Dll3* (up-regulated in *Dll1*^{tm1Gos} mutant embryos), *Hey1*, *Hey2* or *Hes5* were not represented by a probe on either microarray platform. The full expression profiling data is freely available at the Gene Expression Omnibus (GEO) database (Edgar et al., 2002).

1.2. Validation of Dll1 regulated genes by whole-mount in situ hybridization

We used the RNA expression profiling as a screen for the identification of candidate genes for in situ hybridizations. Accordingly we systematically made whole-mount in situ hybridizations with E10.5 wt embryos of all top 47 genes listed in Supplemental Table 1. Those genes with a distinct and reproducible pattern of expression were selected for in situ hybridization with homozygous *Dll1*^{tm1Gos} mutant embryos for the analysis of changes in gene expression patterns. A selection of these is shown in Fig. 1.

Bnip3 (*BCL2/adenovirus E1B 19kDa-interacting protein* 1, *Nip3*) expression at E10.5 was not described (Boyd et al., 1994). In situ hybridization in wt embryos revealed segmented expression along the anterior–posterior axis of the trunk region, the posterior presomitic mesoderm (psm), limb buds and low levels of expression in most cells throughout the embryo. In homozygous *Dll1*^{tm1Gos} embryos the segmented expression was less restricted whereas expression in other domains appeared normal (Fig. 1a,b). *Ddx6* (*DEAD* (*Asp-Glu-Ala-Asp*) *box polypeptide* 6, *HLR2*) encodes an RNA helicase and was expressed in the dorsal neural tube and in ganglia or somitic segments along the trunk of the embryo (Seto et al., 1995). In homozygous *Dll1*^{tm1Gos} embryos expression in the neural tube was reduced but segmented expression was maintained (Fig. 1c,d). *Nes* (*nestin*) is expressed at E10.5 in multipotent neuroepithelial cells in the brain and the neural tube, migrating neural crest cells, and dorsal root ganglia (Dahlstrand et al., 1995; Lendahl et al., 1990; Lutolf et al., 2002). Expression in the neural tube is strongly reduced in homozygous *Dll1* mutant embryos while expression in neural crest cells is still present (Fig. 1i,j). However, segmented expression in neural crest cells is irregular and more restricted in mutant embryos. *Csk* (*c-src tyrosine kinase*) encodes a non-receptor protein tyrosine kinase involved in intracellular trafficking processes (Avrov and Kazlauskas, 2003). At E10.5 expression in branchial arches, trigeminal ganglia, distal limb buds and dorsal root ganglia was detected (Fig. 1k). In *Dll1* mutant embryos expression in the dorsal root ganglia was reduced and the segmented pattern of expression was less restricted, while other expression domains appeared unaffected (Fig. 1 l). The change from a restricted and segmented expression of *Csk* and *Bnip3* in the trunk region of wt embryos to a broader and less restricted domain in mutant embryos, may possibly account for the up-regulation of these genes in the expression profiling experiments. *S100a10* (*S100 calcium binding protein A10* (*calpactin*), *CAL12*, *CLP11*) belongs to the multi-gene family of Ca^{2+}-binding proteins of the EF-hand type (Donato, 1999). At E10.5 expression was observed in neural crest derived cells and cranial ganglia V, VII, IX and X of wt and *Dll1* mutant embryos (Fig. 1m,n). *Sema5b* (*Semag*, *SemG*) is expressed in the neuroepithelium along the entire anterior–posterior axis. In the caudal part of the neural tube the signal is weak (Adams et al., 1996). In *Dll1* mutant embryos expression in the neuroepithelium was strongly reduced (Fig. 1o,p). *Fgf6* (*fibroblast growth factor 6*) is expressed in myotomes between E9.5–E11.5 (de Lapeyriere et al., 1990). In E10.5 homozygous *Dll1*^{tm1Gos} embryos the size of *Fgf6* expressing myotomes was irregular and fusions between segments were observed (Fig. 1q,r).

In addition to the 47 most significant genes, we selected individual genes that were not among the top regulated genes. In particular we noticed the regulation of *Pdgfa* and *Pdgfra*. It appeared to us that the occurrence of these two genes was unlikely to be an artifact. Whole-mount in situ hybridizations subsequently confirmed significant changes